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MALIGNANCY TEST (CANCER TEST) USING THE POLYMERASE CHAIN REACTION

Inventor; and Inventor/Applicant (for US only):	Margit Balazs-Fröhlich [DE/DE] Quellenweg 2 D-5068 Odenthal (DE)
Applicant and Inventor:	Victor Balazs [DE/DE] Quellenweg 2 D-5068 Odenthal (DE)
Agent:	Wulf Bauer Wolfgang-Müller-Str.12 D-5000 Cologne 51 (DE)

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(57) Abstract

In a malignancy test (cancer test), the RNA is concentrated under the constant effect of a reliable RNase inhibitor by in vitro enzymatic multiplication of the specific RNA sequence of the substances of cancer cell origin contained in an acellular biological liquid. The product so obtained undergoes in vitro reverse transcription in order to synthesise the complamentary DNA (cDNA). The product so obtained is used for repeated enzymatic synthesis of the target-specific sequence of the desired substance of cancer cell origin and thus multiplied. The product so obtained is detected by determination of the multiplication of the nucleic acids.

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The invention concerns a method for detecting the specific mRNA sequence (target sequence) of substances of cancer cell origin from an acellular biological fluid in correspondence with the generic part of Claim 1.

The more information is known, and the earlier it is known, the better the patient can be treated. An uninterrupted two-way interaction takes place between the malignant process and the host from the moment the first malignant cell arises.

This mutual interaction is a very complex, multifactor and many-sided mechanism in which the mRNA of cancer cell origin can play an important role, as is listed in detail below:

1. We first reported that the blood plasma RNA fraction of cancer patients in a protein-synthesizing cell-free system functions as a messenger and significantly inhibits the translation activity of other mRNAs, if they are in competition with it.

2. This was later confirmed in RNA transfer (experiments) to normal cells: the translation product of the plasma RNA of cancer patients was produced in the recipient cells, while the production of the corresponding normal protein was drastically inhibited.

This phenomenon is actually the functional mimicry of the primary characteristic of the malignant process itself at the molecular, biological and cellular level: promotion of its own existence at the expense of the host.

3. It was known that malignant cells can release or secrete poly(A)+RNA, protected in a lipoprotein coat or bonded to other substances, and in the overwhelming majority of cases it can be isolated from the blood plasma. Messenger RNA from more one substance of cancer cell origin can occur in the circulation of cancer patients.

4. A broad spectrum of normal and malignant cells is capable of taking up mRNA from the surroundings when it is present protected by lipid, lipoprotein or by other substances. The mRNA then carries out its translation activity in the recipient cells. As a result of this, the translation product of the absorbed mRNA is produced by these cells as long as the supply in the circulation from the surroundings is sufficient.

Thus, the recipient cell has acquired a new phenotyic characteristic, a new production capacity, which it genetically neither has nor can express.

This new biological phenomenon and its importance was first recognized by us and was provided, labeled with the name "phenotype lending."

Phenotype lending is one of the most important mechanisms by which malignant cells achieve their primary goals:

a. promotion of their own existence,

b. prevention (inhibition) of the functions of the host, even

c. the malignant cells compelling the host cells to produce what is advantageous for the malignant process.

The messages that have this effect essentially go in two directions:

1. from the malignant cell population to the host cells.

2. From one subpopulation of the malignant cells to another subpopulation either

a. within the tumor or

b. from the parent tumor cell to far-removed metastasizing tumor cell colonies, and

c. vice versa.

There are a number of published clinical and experimental observations that describe phenomena in malignancy in which said exchange of information through phenotype lending with mRNA can be fully operational:

A. Clinical observations

1. The enzyme profile of the otherwise normal liver of cancer patients is more similar to malignant cells than in the case of normal persons.

2. Normal lymphocytes in the overwhelming majority of patients with myeloma have the myeloma protein as their surface immunoglobulin.

3. Normal leukocytes of cancer patients produce large quantities of enzymes with basal cell destroying activity, which they do not do in a normal state.

B. Experimental observations

1. A subpopulation of malignant cells that is not capable of metastasizing when it is injected s.c. will, however, metastasize after a subpopulation with metastatic potential of the same tumor has been injected i.v. into the same animal.

2. A subpopulation of malignant cells was resistant to a drug with which it had never previously been in contact when only humoral contact was established between this subpopulation and another subpopulation that was resistant to this drug.

This list is not exhaustive.

Here it must be noted that the transcripts or their fragments of cellular oncogenes that are activated by hypertranscription in malignant diseases can be detected from the blood plasma of cancer patients by in vitro hybridization. Phenotype lending by these transcripts can give rise to similar phenomena to A1 and B1 in malignant diseases or can transfer the ability to produce growth factors, enzymes or other substances in host cells that are at a distance from the tumor. Thus, the environment of these host cells may be attractive for the arriving metastasizing cancer cells and may then feed these cells with growth factors and other substances. Phenotype lending by mRNA of malignant origin inhibits at the same time the characteristic functions in the recipient cells of the host, which can lead to a reduction of the local and systematic defense

mechanisms of the host. Small fragments of the transcripts of cancer cell origin also have inhibiting activity.

The occurrence of phenotype lending via the circulating mRNA was investigated and demonstrated in myeloma patients (A2, see list) by us and others.

We were successful in transferring the production of the myeloma protein into normal human lymphocytes with the plasma RNA from myeloma patients, which also gave rise to a reduction of the production of characteristic surface immunoglobulin. The RNase-treated sample of the same plasma RNA was without effect.

The RNA transfer process, however, is a very complicated laborious method and is not sensitive enough to give positive results in the very early stage of malignancy.

The specific mRNA sequence of the H and L chains, corresponding to the chain combination of the relevant myeloma protein, was detected in the blood plasma of myeloma patients. The presence of the mRNA in the bloodstream is the most important prerequisite of phenotype lending. Therefore, the detection of mRNA or the specific substances of cancer cell origin in the bloodstream of cancer patients in accordance with the methods of the invention can serve as a sensitive and quite practical test method that can provide important information on the underlying fundamental molecular genetic changes in the malignant cells, their activities, the possible interaction between tumor and host through mRNA even in the early stage of malignant diseases, at a point in time when the cancer cell mass cannot be visualized by conventional methods and is not accessible for histological examination.

The circulating mRNA transcripts or their specific fragments can be detected with very sensitive and specific methods. One technical possibility of this kind is in vitro hybridization and another is in vitro enzymatic amplification of the specific target sequence of the relevant mRNA by the polymerase chain reaction (PCR).

The polymerase chain reaction, which has been known since 1985 (Science 230: 1350-1356), is a reliable, relatively simple method for synthesizing and amplifying in vitro a desired sequence of DNA that is present in a low concentration, from tissue, cells or even from a single hair. The amplified product can then be easily identified by a broad spectrum of various methods including in vitro hybridization with the appropriate labeled DNA or oligonucleotide probes. For instance, PCR has been used to track and to identify spots or residues of cellular body fluids (blood, semen) or body cells.

The mRNA sequences can also be amplified in vitro by PCR if the corresponding complimentary DNA (cDNA) is first synthesized by reverse transcription and amplified by PCR. This RNA amplification was previously used only for cells and cellular body fluids like blood, in particular to identify RNA viruses in blood cells. Two oligonucleotide primers that are complimentary to the sequences that flank the desired specific sequence are used in PCR, one

primer for the 3′ strand and one primer for the 5′ strand, which "mark" the sequence that the DNA polymerase enzyme is supposed to synthesize and amplify.

The target-specific sequence can be amplified by PCR to an extent high enough that direct detection of the product is possible. If labeled structural elements or primers are used, the PCR products can be visualized through their labeling and do not have to be detected by in vitro hybridization. PCR has as many advantages as in vitro hybridization, for instance, high specificity, very high sensitivity, which is on the same level or an order of magnitude higher than in vitro hybridization, and it is simpler, faster and can be automated. This is why PCR is especially suitable for detection of the mRNA sequence of the substances of cancer cell origin from an acellular biological fluid like blood plasma and can serve as a sensitive malignancy test for diagnosis, early detection and monitoring of malignant diseases.

Detection of the mRNA transcripts or their specific fragments of the substances of cancer cell origin as a malignancy test has many advantages over the detection of the substances themselves:

1. The sensitivity of the mRNA detection method in accordance with the invention is considerably greater (lies in the region of 10^{-18} g) than the methods of detecting the translation products, thus the substances themselves (lies in the region of pg).

2. Antibodies to the translation products, either produced by the hosts or introduced (inserted) for purposes of therapy, do not affect the test results in the mRNA transcript detection.

3. RNA transcripts or their specific fragments are released or deposited in detectable amounts and in a form protected against degrading enzymes only by malignant cells. This is why their detection (in the bloodstream) from blood plasma is diagnostic, in contrast to their translation product, which can also be produced by normal cells (for example: oncogene products; some tumor markers).

4. The half-life of the mRNA transcripts is considerably shorter (60 min for onc-fos, onc-myc; 7 h for CEA) than their translation products (several days for oncogene products, more than a week for CEA).

This results in important practical advantages for the method of detecting mRNA transcripts or their specific fragments that can be used with the method of the invention as a malignancy test:

a. Any changes can be recognized considerably earlier and faster;

b. The effect or lack of effect of treatment can be observed and evaluated more quickly;

c. The treatment outcome of any chemotherapy or immuno(chemo)therapy can be foreseen in good time in only one to two days. Tests with malignant cells showed that effective chemotherapy leads to a considerable reduction even up to completely stopping the transcription of the activated oncogenes. Then mRNA transcripts or their fragments of activated oncogenes

will no longer be present in the blood plasma, or they will be present only in a reduced concentration. If the oncogene-specific mRNA sequences continue to persist in the bloodstream without change, this means that the chemotherapy being conducted is ineffective. Thus, the unnecessary danger of a subsequent chemotherapy that has already proven ineffective can be avoided ahead of time and a new combination of antineoplastic agents can be "tailor-made" quite individually for each patient.

d. The development of resistance through multidrug resistance factors can be observed still earlier and can be appropriately treated.

5. The detection of new tumor markers or cancer substances that are produced in cancer patients even by their normal cells, if the mRNA transcript or the specific fragments of these substances are detectable in the circulation, in the blood plasma, the ability to produce these substances is then not just the characteristic properties of the host cells, but rather the consequence of "phenotype lending," through the circulating mRNA coming from the cancer cells and the property thus acquired.

Thus, the appearance and the reality can be brought into correspondence. There are phenomena in which the effect (this effect) can be seen at first glance: production of the myeloma protein as a surface immunoglobulin by the otherwise normal lymphocytes of the host. However, there are also phenomena in which this is not immediately recognizable: the case of an enzyme that is produced simultaneously by the cancer cells and at the same time by the otherwise normal liver in cancer patients.

These instances can be clarified through the detection of the corresponding mRNA or its specific fractions in the circulation.

6. Finally, a number of therapeutic possibilities result from the knowledge of the circulating mRNA profile. Through the inhibition of the production, deposition or translation of the corresponding mRNA of cancer cell origin, a disadvantageous effect for the host or an advantageous effect for the malignant cells can be pushed off and avoided very early, for example, via an antisense oligonucleotide, and so forth.

a. In plasmacytoma the prevention of the transfer of the myeloma protein production capacity to the normal lymphocytes of the host in an early stage of the malignancy can prevent the inhibition of the immune system so much that the immune system can eliminate the relatively small tumor cell mass without further chemotherapy if the malignancy was recognized early and treated in accordance with the possibility noted above.

b. Through the inhibition of the endothelial-basal membrane destroying enzymes, the tumor cells present in the bloodstream cannot leave the circulation and thus cannot metastasize. This is especially important during and after surgery, as a larger number of cancer cells can get into the bloodstream.

c. The lower (20-25%) success rates of the various forms of immune therapy can be at least partially explained by phenotype lending, since because of this the host cells cannot sufficiently act together or the cells amplifying and activated in vitro can become inhibited in their function shortly after being administered to the cancer patient (host), without effect.

d. Through removal of the lipoprotein-mRNA complexes, for example, by hemosorption similar to what is already done to remove harmful lipids in certain forms of hyperlipidemia, or by other suitable methods (plasmapheresis), in the early stages of the disease, the harmful effect on the host by the malignant cells could be interrupted and the host could then destroy the malignant cells with full strength.

If the in vitro amplification of the specific mRNA sequences is combined with in vitro hybridization, the sensitivity of the malignancy tests increases by at least 4-5 orders of magnitude when compared to hybridization alone or PCR alone.

In clinical oncology, early means a cure in at least 80-90% of cases. Therefore, any method that can significantly improve early recognition of malignant processes from the standpoint of time will be welcome in clinical practice.

A jump of several months in early recognition and thus several vital months for early introduced therapy can thus lead to complete cure. This is the most important advantage of this invention.

The activation of two or more cellular oncogenes through elevated transcription or through mutation is generally interpreted as a very important step in the development, maintenance and progression of the malignant transformation. This elevation of transcription can reach many times, even 100 times the normal values. Because of the special properties of malignant cells and malignant tumors, the activated oncogene transcripts or their fragments can get into the bloodstream and can be present there in protected or partially protected form without further significant degradation, and they can thus be detected in the bloodstream of patients with an activated malignant process by a sensitive method such as in vitro hybridization. This method can be used as a malignancy test and is suitable as a screening test or monitoring test in order to detect relatively small amounts of malignant cell masses that cannot be seen with the conventional diagnostic methods in clinical practice.

With the method of this invention one can, for example, detect the oncogene-specific mRNA sequences in the bloodstream of even such oncogenes that are present there in a still lower concentration than others, for example, the transcripts or transcripted fragments of the oncogenes that are activated by point mutation and not by elevated transcription, and for this reason, their concentration is lower in the malignant cells and subsequently also in the blood plasma in comparison to oncogenes that were activated through elevated transcription and even in vivo amplification. Thus the plasma oncogene profile (oncogenes whose mRNA sequence is

present in the blood plasma) and the cancer cell oncogene profile (oncogenes that are activated in malignant cells) can be reflected better. Thus, one could not only [detect] the presence of an active malignant process from the bloodstream, but one could also predict which oncogenes are at least partially activated in the malignant cells, and already at a point in time at which the malignancy cannot yet be visualized, and thus is not yet accessible for histologic and molecular/genetic tests.

Over 50% of adenomas with precancerous changes in the large intestine have ras oncogenes activated by point mutation (particularly Ki-ras), while cancerously changed adenomas have these oncogenes in a somewhat lower percentage. The mutation appeared most frequently with the Ki-ras oncogenes in the position of codon 12, and GGT is mutated to GAT in both conditions. The malignancy tests conducted without prior amplification of said mutated oncogene sequence from blood plasma RNA gave a negative result in both conditions, while after in vitro amplification, the malignancy test was positive only in the case of cancer, but not in the case of the precancerous adenomas. This example clearly shows that the invention, in spite of its increased sensitivity, is a reliable malignancy test.

The goal of cancer research and clinical oncology is therapy that is causal, rational and meaningful at the molecular and genetic levels. In this regard, the knowledge, and especially the early knowledge, of which oncogenes are actually activated in the malignant cells is very important. These oncogenes can serve as a good target of a molecularly and genetically meaningful therapy, and specifically, they can be attacked at several levels, namely (a) at the gene level: by preventing transcription; (b) at the transcript level: by preventing translation of activated oncogenes; (c) or at the oncogene product level: by preventing the functioning of the OKPs [expansion unknown; possibly oncogene products]. Very stimulating results with cancer cell cultures in vitro are known for (a) and (b).

An oligonucleotide with a specific sequence for the initiation of protein synthesis is preferably taken up by the cancer cells in larger amounts than by normal cells and inhibits the translation of the targeted oncogene RNA transcripts with the result that the cancer cells lose their properties and no longer malignantly multiply. This possibility is a good candidate for developing a causal therapy in practice.

Experiments with malignant cells or with tumors carried out with monoclonal antibodies in animal experiments demonstrated that, as long as the OKP is neutralized by specific antibodies, the malignant phenotype disappears. These examples clearly show that the molecularly and genetically causal meaningful therapy is in principle possible and it is not conceivable without the early knowledge of which oncogenes are activated in the malignant cells. The oncogene profile of the blood plasma is the method of this invention by its early help here.

If the anticancer cell antibodies are bonded to chemotherapeutic, toxic or radioactive substances, therapeutic activity can be achieved in animals and patients, but the success rate is generally not very high. Several factors are responsible for this, one of which is the differences in the expression of the targeted antigen at the various cancer cells of the same tumor because of their diversification. Better results can be achieved if a number of antigens at the cell surface are simultaneously attacked in an immunologically targeted way. The oncogene products of the activated oncogenes would be especially suitable as such antigens, especially ones that have their oncogene product in the cell membrane and ones that do not occur at all in normal cells, for example, the oncogene products altered by point mutation or certain tumor markers. Another advantage is that the oncogene transcripts or their sequences can be detected much earlier than is possible with the most sensitive immune methods.

The immunological detection of oncogene products is not suitable as a malignancy test since the normal and pathological values (in malignancies) overlap each other, and some abnormal, but nonmalignant, conditions produce higher values than malignancies, even apart from the considerably lower sensitivity of the immunological methods in comparison to the current invention.

Another important advantage is changes in the activation of oncogenes during monitoring can be reflected even more reliably and earlier through the plasma oncogene profile than with the current methods. Thus, oncogene changes that are connected to a deterioration of the prognosis due to diversification of the cancer cells or with an amplification of one of the activated oncogenes can be caught early and the appropriate therapy can be introduced in a timely way. The disappearance of an oncogene transcript that is present or the appearance of a new oncogene transcript or its sequence that was not present in the blood plasma is also a sign of worsening progression. If the ratio of 2 or more oncogene transcripts to each other changes significantly in the blood plasma during monitoring, this can reflect amplification in vivo of one of the oncogenes, which in most cases could mean an increase of the aggression and increased progression of the malignancy.

The mechanism of the phenomena "phenotype lending" practiced by nature can be imitated and employed for therapeutic purposes through the administration of mRNA lipoprotein complexes in order to

1. replace the lacking production of substances,

2. increase the production of one or more substances and thus to combat a pathological condition,

3. or to enable the production of such substances that do not occur in the human organism, but that would be therapeutically advantageous.

These possibilities for therapy have the great advantage that the effect is controllable and can be stopped at any time by stopping the administration, in contrast to genetic transfer with DNA, which for many reasons is impractical anyway in humans.

The possibility of therapy with mRNA, however, requires careful monitoring of the mRNA that is used or its specific sequence in the bloodstream. The method of the invention is very suitable for this purpose because of its very high sensitivity and reliable specificity.

The invention is based on the problem of detecting specific mRNA sequences of the substances of cancer cell origin from an acellular biological liquid even when the substances or their fragments are present in a very low concentration and have been degraded to a certain degree, but have the specific sequence and are suitable for in vitro amplification, and this method is thus to be used as a malignancy test, especially as a very sensitive early detection test.

This problem is solved by the method as in Claim 1. Through the use of an effective and reliable RNase inhibitor, but one that does not give rise to the escape of RNA from cells, even in sampling the cellular biological liquid, the degradation of the RNA or its fragments is prevented. Since the selected RNase inhibitor does not cause any escape of RNA from the cells before or during their removal, it is important because this sensitive method can already detect a small contamination.

The PCR products can be radioactively or nonradioactively labeled during PCR by incorporation of labeled structural elements or primers and can be visualized in this way. The nonlabeled PCR products can be identified by direct detection after ethidium bromide staining or by their size, or in correspondence with their sequence either by sequence analysis or especially with in vitro hybridization.

Thus, a high degree of sensitivity, an extremely high specificity, reliability and reproducibility are achieved. Because of the special properties of malignant cells and tumors, the method is suitable as a malignancy test, especially for early detection.

The invention is explained in more detail below. The cellular biological fluid (such as blood, exudates, etc.) is mixed with a reliable RNase inhibitor that does not give rise to RNA-cell leakage during sampling, and the cells are removed. From the resulting acellular biological fluid, the total RNA is mixed with an aqueous medium under the constant effect of the RNase inhibitor and in the presence of a detergent and a proteolytic enzyme like proteinase K, and incubated in a buffer solution at 37°C for 5-8 h. Then yeast tRNA is mixed in as a carrier (50 μg/mL) an extraction is carried out with phenol and chloroform at 60°C, and precipitation is carried out with alcohol at -20°C. The extracted material can still be quickly purified further by ion-exchange column chromatography.

The blood plasma RNA is mixed with a buffer solution that is composed in correspondence with the optimum mechanism of the selected reverse transcriptase (Tris Cl

[sic; HCl], pH 8.3, KCl, $MgCl_2$), and it additionally contains RNasin, reverse transcriptase enzyme, each of the four deoxyribonucleoside triphosphates, and the 3′ primer, all in sufficient concentration. Then the total reaction mixture, 20 μL, is incubated at 37°C for 30 min.

After that the reaction mixture is mixed with 80 μL PCR buffer and 100 μg of bovine serum albumin, the 3′ primer and the 5′ primer, and the thermostable Taq DNA polymerase enzyme, each in an appropriate concentration. The reaction mixture is then covered with mineral oil to prevent evaporation. By heating the reaction mixture to 95°C for 20 sec, the RNA-cDNA complex is dissociated, and annealing of the primers is carried out at 55°C for 15 sec. The extension of the primers takes place at 72°C for 1 min. By heating, the cycle is restarted and is repeated 40-50 times.

After the last cycle, the reaction mixture is left for 10 min at 72°C and then cooled on ice.

The occurrence of amplification during PCR is detected by ethidium bromide staining in comparison to a negative control. The product or products can be identified by size by means of gel electrophoresis, or by chromatography, or in correspondence with their sequence.

Products can be labeled with a radioisotope, biotin, enzymes, fluorescent dyes or other substances during the amplification by means of labeled structural elements or primers and are then recognized in correspondence with the labeling.

Identification of the PCR products with the maximum sensitivity and with maximum specificity is achieved by in vitro hybridization.

10 μL of each reaction mixture are applied to nylon or nitrocellulose filters and immobilized by baking at 80°C for 60 min, prehybridized in a suitable buffer at 60°C and then hybridized in the presence of 2×10^6 cqm [sic; cpm] end-labeled oncogene-specific oligonucleotide probes per mL for 5-7 h at 60°C. Washing is carried out at 60°C in 0.75M NaCl/0.075 [sic] Na citrate and 0.1% SDS. The result of hybridization is visualized by autoradiography.

In a variation of this method, which is actually its mirror image, the products of the simultaneously pooled PCR are identified at the same time. The unlabeled corresponding probes are immobilized on a solid carrier like a nylon filter and brought into contact with the (biotin) labeled PCR products in order to hybridize them with each other. The results are visualized by enzyme affinity-related color reaction.

A series of purified molecularly cloned DNAs that contain the sequence as codes of the oncogene are labeled either radioisotopically or nonradioisotopically (J. Mol. Biol. 113: 237-251, 1977). The nonradioisotopic labeling can take place with biotin. In the case of oligonucleotide probes, it is carried out by end labeling in order to achieve high specific activity.

DNA probes and oncogene-specific oligonucleotide probes, labeled or unlabeled, are commercially available (Oncor, Inc., Gaithersburgh, MA 20877, USA; Oncogene Science Inc,

Mineola, NY 11501, USA; Amersham, Little Chalfont, England). A biotin labeling and visualization kit is sold commercially (Amersham, Little Chalfont, England; Oncor, Gaithersburgh, USA). Oligonucleotides with the relevant desired sequences can be ordered from custom services in Europe and in the USA.

Every measure was undertaken to prevent contamination with the relevant target sequence before, during and after the research processes.

The danger of mixing in the ubiquitous, highly resistant RNase enzyme from exogenous sources is especially reduced in all steps of the RNA phase of the method (Steps a through c) by using baked glassware, autoclaved solutions, baked spatulas and tools, dry chemical preparations, glass distilled autoclaved sterilized water, and wearing gloves.

The following example serves for further illustration of the invention, without limiting it.

Example

10 mL blood is withdrawn with 20 IU heparin and immediately mixed with a solution of RNase inhibitor like RNasin (Promega Biotec, Madison, WI, USA, end concentration 2000 U/mL). The blood plasma is separated as quickly as possible. Then 2.5 mL blood plasma is pipetted into each of two single-use plastic centrifuge tubes, one of which is deep-frozen for a possible repetition or stored for supplemental tests as a reserve. The other is immediately taken for processing.

A mixture of 0.2M Tris Cl (pH 8.5); 25 mM ethylenediaminetetraacetic acid (EDTA); 2% (w/v) sodium dodecylsulfate (SDS) and proteinase K (end concentration: 300 μg/mL) is added and the mixture is incubated in the presence of RNase inhibitor at 37°C for 5-8 h.

Then yeast tRNA (50 μg/mL) is mixed in, followed by an equal volume of phenol and chloroform-isoamyl alcohol (24:1) and extraction is carried out at 60°C for 15 min by vigorous shaking. The intermediate phase is additionally extracted one more time as above, and the combined aqueous phases are treated one time with chloroform-isoamyl alcohol as above, but at room temperature. The nucleic acid is precipitated with alcohol in the presence of Na acetate (0.3M, pH 5.2) at -20°C and centrifuged. The sediment is dissolved and subjected to an RNase-free DNase enzyme treatment.

In this case, RNase-free DNase enzyme and $MgCl_2$ (to 2 mM) is mixed into the plasma RNA solution (50 mM Tris Cl, pH 7.5 in 1 mM EDTA) and incubated in the presence of RNase inhibitor at 37°C for 30 min, then extracted with phenol-chloroform, and in the presence of Na acetate (pH 5.2, 0.3M), the RNA is precipitated with 70% ethanol at -20°C.

An additional purification process by ion-exchange column chromatography ("The Extractor," Molecular Biosystems, San Diego, CA 92121) can be carried out in about 30 min in correspondence with the manufacturer's instructions before the DNase treatment.

When the extraction of the RNA from the RNA lipoprotein fraction is carried out, one obtains an RNA fraction that preferably contains poly $(A)^{+}$RNA. The RNA lipoprotein fraction can be separated from the serum of cancer patients by flotation in a KBr density gradient (discontinous: 1.006-1.221 g/mL), and after 16 h flotation in an ultracentrifuge at 105,000 G, it becomes visible as an opal band between the HDL and LDL fractions.

The eluate can be used directly for in vitro hybridization or for PCR amplification, or after alcohol precipitation and DNase treatment.

Then the sediment is [transferred] to a buffer solution that corresponds to the optimum activity of the reverse transcriptase enzyme that is used (and the instructions of the relevant manufacturer) (Bethesda Research Laboratories; Promega Biotec, USA) (50 mM Tris Cl, pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 1 nM dithiorheitol [sic; dithiothreitol], 1 U RNasin). The solution is supplemented with 1 mM of each deoxyribonucleoside triphosphate, 1000 U reverse transcriptase, and 10 pM of the relevant 3′ PCR primer to make 100 μL.

20 μL of this reaction mixture is incubated for each target sequence or each pooled target sequence system at 37°C for 30 min.

80 μL PCR buffer (50 mM Tris Cl, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg bovine serum albumin/mL, pH 8.4, 40 pM 3′ primer, 50 pM 5′ primer and 1 U thermostable Taq DNA polymerase (Perkin-Elmer-Cetus, USA; New England Biolab, USA)) are mixed into this reaction mixture. Mineral oil is used to cover the reaction mixture in order to prevent evaporation.

By heating to 95°C for 20 sec, the RNA-cDNA complex is dissociated and annealing of the primers takes place at 55°C for 15 sec, extension of the primers takes place at 72°C for 1 min. The cycle is restarted by heating and is repeated 40-50 times.

After the last cycle, the reaction mixture is left for 10 min at 72°C and then cooled on ice. The "thermal cycler" instrument (Perkin-Elmer/Cetus, USA) considerably simplifies reliable and rapid conduct of PCR.

Primer pairs to synthesize PCR products of different sizes.

An example:

Primer Pair for Ha-ras12	Sequence	Size of amplified product in base pairs (bp)
5′ primer (sequence)	GACGGAATATAAGCTGGTGG	63
3′ primer (sequence)	TGGATGGTCAGCGCACTCTT	
Primer Pair for Ki-ras12		
5′ primer (sequence)	GACTGAATATAAACTTGTGG	108
3′ primer (sequence)	CTATTGTTGGATCATATTCC	
Primer Pair for Ki-ras61		
5′ primer (sequence)	TTCCTACAGGAAGCAAGTAG	128
3′ primer (sequence)	CACAAAGAAAGCCCTCCCCA	

Labeling of the PCR product is carried out during amplification by incorporating either labeled deoxyribonucleoside triphosphate or labeled primers. The labeling can be radioactive or nonradioactive, for example: biotin, enzymes, dyes and other substances. Fluorescent dyes found advantageous use as labeling for primers: these dyes, a different dye for each amplified sequence, are conjugated to the oligonucleotide primers (Applied Biosystems, Foster City, CA, USA).

Detection of the PCR products, or the establishment of the occurrence of amplification, is possible by several procedures:

Direct method by ethidium bromide staining: the unincorporated structural elements and primers must be removed first. The reaction mixture is diluted to 2.5 mL with 10 mM Tris Cl, pH 8.0, loaded into a Centricon -100 microfiltration tube (Amicon) and centrifuged at 5000 rpm (Sorvall SS35, fixed angle rotor). The amplified DNA can be recovered from the upper part of the filter (45-50 μL). About 5 μL is then applied to an agarose plate that contains 0.5 μg/mL ethidium bromide and visualized by fluorescence induced by UV transluminator in a comparison with a negative control (the same composition minus plasma RNA, same treatment) and evaluated.

1. Labeled PCR products are identified by their labeling. In this case, the unincorporated labeled elements are removed (as above).

a. Radioactively labeled PCR products are determined by measuring the radioactivity compared to a negative control, or visualized by autoradiography after agarose gel electrophoresis without prior treatment and even identified by the size of the product.

b. Biotin-labeled PCR products are detected after eliminating the unincorporated, labeled elements by enzyme affinity related color reaction. Visualization kits are commercially available (Oncor, Gaithersburgh, MD, 20884, USA; Amersham, Little Chalfont, England).

c. PCR products labeled with fluorescent dyes are identified by their color with a fluorometer (Perkin-Elmer LS-5).

2. Detection of unlabeled products by their size:

There are two technical possibilities available here. Prior treatment is not necessary:

a. Electrophoresis in agarose or acrylamide gel, in correspondence with the size of the products. Visualization either by EB fluorescence or in correspondence with the labeling. The size of the products is determined by comparison to parallel running markers with known size.

b. Ion-exchange chromatography (high resolution separation): The reaction mixture after amplification is input to a monoQ HR 5/5 column (Pharmacia, Uppsala, Sweden) and eluted through the given gradients of buffer solutions A and B at a flow rate of 0.15 mL/min. Buffer solution A: 20 mM Tris Cl, pH 8.3, 0.4% M [sic] NaCl; buffer solution B: A + 1.0M NaCl. Gradient: 40-60% B in 2 h and 60-80% B in 6 h.

Simultaneous multitarget sequence PCR (pooled PCR).

This method gives rise to special problems for the identification of a plurality of PCR products amplified in the same reaction mixture in a single tube. In this form of PCR, more than one sequence, either of the same molecule or fragment, or sequences of a number of different mRNAs or their fragments are simultaneously amplified if the corresponding primer pairs are present in sufficient concentration.

In this case, it is shown with EB fluorescence whether amplification has taken place, but the sequence or sequences that were amplified must be determined with special additional measures. In the case of the unlabeled products, identification is achieved via the product size. For this, the size of the various PCR products is programmed beforehand through the choice and use of the appropriate primer pairs so that the product of each amplified target sequence is synthesized and amplified in an individual, characteristic, different size during the PR [sic; PCR] (see example). PCR products can be identified by their size either by gel electrophoresis or ion-exchange chromatography.

The second possibility is differentiation of the products by color if they were labeled differently with fluorescent dyes, each with its own color.

3. The third possibility for differentiation of simultaneously amplified products is identification via their sequence. There are three solutions for this which are also suitable and necessary for monitoring the authenticity of the Taq polymerase activity due to "misreading" that occurs and their amplification:

a. Sequence analysis is especially suitable for testing the authenticity of the enzyme activity.

b. In principle, scanning tunneling microscopy is a possibility for identifying the PCR products. It is currently possible to differentiate only the purine and pyrimidine bases from each

other with this type of microscopy. Through improvements to the resolving power of the microscope it will become possible in the near future to identify the different bases, even from a concentrated RNA or DNA extract.

c. Hybridization in vitro is the most practiced method for identifying nucleic acids in correspondence with their sequence, specifically in its conventional form such as dot, slot, or Southern blot. In this form, the PCR product is immobilized on a solid substrate and is brought into contact with labeled specific oligonucleotide or DNA probes that are free in solution in order to hybridize with each other when a complimentary sequence is present. The nonspecifically bound probes are removed by washing.

From each reaction mixture 20 μL is applied with a dotting apparatus under the effect of a mild vacuum to a nylon or nitrocellulose filter that has been prewetted with 20x SSPE, then the filters are washed in a small volume of 20x SSPE and immobilized by baking at 80°C for 60 min. Then the filters are prehybridized in a solution of 0.75M NaCl, 0.075M sodium citrate, pH 7.0, 20 mM Na phosphate, pH 7.0, 5 mM EDTA, 200 μg/mL yeast-tRNA, 1% Sarkosyl (Sigma) for 60 min at 60°C. The buffer in which the prehybridization took place is removed and a buffer of the same composition that additionally contains 2 x 10^6 cpm/mL 5′ end-labeled denatured oligonucleotides with oncogene-specific sequence or with the sequence that contains the mutated codon of an oncogene (like ras oncogenes) is mixed in, and incubation is carried out at 60°C for 6-8 h for the hybridization to take place. Then the filters are washed in a solution of 0.75M NaCl, 0.075M Na citrate, and 0.1% SDS at 60°C. Visualization of the occurrence of the hybridization is achieved by autoradiography with the help of an amplifying shield.

In the case of oncogene-oligonucleotide probes, which conduct the sequence with the mutated codon (like Ki-ras, Ha-ras, N-ras, codon 12, 13, 61) it is more advantageous if the buffer solution for prehybridization, hybridization and washing additionally contains tetramethylammonium chloride (3M tetramethylammonium chloride, 50 mM Tris Cl, pH 7.5, 2 mM EDTA, 0.3% SDS, 100 μg/mL sonicated salmon sperm DNA, 5x Denhardt's solution (1x Denhardt's solution: Ficoll, polyvinyl pyrrolidone, bovine serum albumin, 0.02% each). The filters in this case are then washed two times in 2x SSPE (1x SSPE: 10 mM Na phosphate, pH 7.2, 0.18M NaCl, 1 mM EDTA, 0.1% SDS) for 10 min at room temperature, then they are rinsed with 3M tetramethylammonium Cl hybridization buffer minus the carrier DNA and Denhardt's solution and then washed in the solution for 30-60 min at 60°C. The filters are evaluated by autoradiography as above.

The following known 18 molecularly cloned human oncogenes or their oncogene-specific sequences can be used as probes. The oncogenes are grouped below roughly according to the frequency of the occurrence of elevated activity in human malignancies:

Group A: fos, myc, Ha-ras, Ki-ras

Group B: fes, myb, fms, N-ras, src, abl, raf, Erb^B, N-myc

Group C: Erb^A, mos, sis, alu, bcr.

The oncogene probes are radioisotopically labeled, mostly with ^{32}P (specific activity: 10^8-10^9 cpm/μg) or are labeled with biotin. Oncogene oligonucleotide probes, including ones that have sequences modified by point mutation of oncogenes activated by point mutation, are radioisotopically labeled by end labeling in order to achieve high specific activity. If new oncogenes are discovered, additional oncogene oligonucleotide probes that can be used in correspondence with the diagnostic purposes might be necessary.

There is an in vitro hybridization process that is the mirror image of the conventional method noted above. In this method, the unlabeled different oligonucleotide probes are immobilized on a piece of nylon filter and the filter is hybridized with PCR products that have been obtained during the amplification and then labeled with biotin; reverse hybridization method.

100 μL oligonucleotide probe solution (100 pmol; in 10 mM Tris Cl, 0.1 mM EDTA) is applied to a nylon filter (membrane) (Genetrans-45, Plasco, Woburn, MA, USA) and immobilized by UV light or by baking, then is [subjected] to a wash with 200 mL 5x SSPE and 0.5% [sic] for 30 min at 55°C in order to remove the unbound oligonucleotide. One filter has a number of different oligonucleotide probes.

Each filter with the different specific oligonucleotide probes immobilized on it is [sic; verb omitted?] in 4 mL hybridization solution consisting of 20 μL of the amplified labeled (biotin) DNA and 5x SSPE, 0.5% SDS. Beforehand, the amplified DNA was [verb missing] by mixing 400 mM NaOH, 10 mM EDTA and was thus rapidly added to the hybridization solution. Then incubation was carried out at 55°C for 4-6 h. The [sic; word omitted?] are rapidly washed with 2x SSPE, 0.1% SDS at room temperature, one time with the same solution at 55°C for 10 min and [subjected] to a short rinse two times in 2x PBS (1x PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) at room temperature. Visualization in correspondence with labeling.

Oligonucleotides that are coupled with a poly dT tail (several hundred bp long): via this tail, the probes are then immobilized on a piece of nylon filter by UV light so that the specific sequence or sequences of the fragment is or are free in solution and thus the hybridization takes place rapidly.

The point mutation possibilities of some oncogenes are numerous and require rapid investigation methods. One possibility is the simultaneous pooled sequence PCR and the differentiation of the various products by different fluorescent dye labeling, through the use of labeled primer pairs, one primer of which is labeled with a different dye and is complimentary to

the mutated sequence. Every 5-6 mutated sequences is indicated by its own dyes and its own color. Thus, in a pooled PCR system, the amplification of these mutated sequences can be identified rapidly and easily.

The PCR cannot be carried out in the case of substances whose nucleotide sequence is not known. In these cases in vitro hybridization with the corresponding DNA probes is the method of choice.

The plasma RNA concentrate was denatured by incubation at 65°C for 15 min and then by rapid cooling. After that, the RNA was applied with a dotting apparatus (Schleicher & Schuell, Keene, New Hampshire, USA) to a solid carrier like nitrocellulose paper, which had been equilibrated beforehand with 20x NaCl/cit. and then dried. Then the thus treated nitrocellulose paper was baked for 2 h at 80°C in a vacuum oven, and incubated after baking, in a solution (prehybridization buffer): formamide (50% (v/v) 5x NaCl/cit; 50 mM sodium phosphate, pH 6.5; sonicated denatured salmon sperm DNA (250 μg/mL) and 0.2% each bovine serum albumin (BSA), Ficoll and polyvinyl pyrrolidone for 6-8 h at 55°C.

The biotin labeled oncogene and other probes (labeled probes or labeling kit: Oncor, Gaithersburgh, USA) are first denatured and then added to the prehybridization solution, which contains the nitrocellulose paper with the plasma RNA immobilized on it. The concentration of the probe or probes (pooled probes) is high: 600 ng/mL. In the case of pooled probes of 5 components, 120 ng/mL each. Hybridization at 55°C for 7-10 h.

The occurrence of hybridization is detected with the visualization kit in correspondence with the manufacturer's instructions (Oncor, Gaithersburgh, USA) after the prescribed washings.

The use of biotin labeled probes and visualization kits from Oncor enables rehybridization after removal of the hybridized probes.

With the pooled probes (5 each) the plasma RNA with the oncogenes most frequently activated in human malignant diseases can be tested relatively quickly and a negative or positive test result obtained. The positive pool is then tested additionally with its components (the individual probes) by hybridization and/or rehybridization in order to determine the plasma oncogene profile.

The sensitivity of this method lies in the range of 0.1 pg. There are numerous additional modifications that are possible.

The intensity of the black spot can be quantitatively measured by autoradiography by the intensity of the color and thus, quantitative comparisons can be made.

Quantitative test:

Serial dilution is made from the plasma RNA, with 10 μg/mL yeast tRNA as diluent carrier RNA, then with each dilution, the reverse transcription is carried out first, then the PCR,

and the product is identified by in vitro hybridization with the corresponding specific oligonucleotide probes by other methods. The highest dilution from which a positive signal is still obtained is the titer. In this way, quantitative comparisons can be carried out during the observation time in the monitoring of a patient. In this way, amplification of one of the activated oncogenes, for example, can be observed. During monitoring, if the titer of one oncogene becomes disproportionately elevated in comparison to other oncogenes that can be detected in the plasma, this can mean the in vivo amplification of this oncogene, among other things.

This method is especially suitable for screening persons who are in particular danger of cancer, such as people exposed to X-rays or radioactive radiation, workers in chemical or asbestos plants, patients with compromised immune system (after chemotherapy) or family members with genetically conditioned tendency for various malignant disease.

Because of the increased sensitivity of this method, there may be nonmalignant cases in which the malignancy test gives a positive result. Differentiation is possible through monitoring. In nonmalignant cases the positivity temporarily persists, and then tapers off, in contrast to malignant cases where positivity persists and then rises.

The simultaneous amplification of a number of target sequences simplifies the investigation of a large number of substances like oncogenes, or variants from point mutations. Example: three PCR systems (pooled PCR), each programmed for 5 target sequences, of which two are for the most commonly activated oncogenes, and the third is for other sequences of cancer cell origin. EB staining shows in which system the amplification has taken place and the amplified sequence or sequences are then identified in correspondence with their size, if they were so programmed, by chromatography or gel electrophoresis.

The test is even faster if the fluorescent dye labeled primer for each sequence to be synthesized in the pool has its own color and the amplified product or products are identified with a fluorometer after the removal of the unincorporated labeled primers without greater delay in correspondence with their color.

The same thing can be done with the point-mutated sequences. Each mutated sequence in the pool is indicated with its own color by a fluorescent dye labeled primer that is complimentary for the mutated codon region.

The most sensitive test method is when the PCR products are detected by in vitro hybridization. In the case of pooled PCR, the reaction mixture after amplification is hybridized with the correspondingly labeled oligonucleotide or pooled DNA probes. The positive pooled PCR mixture is then further hybridized with labeled individual oligonucleotide or DNA probes in order to find out which sequence was amplified in the pool. In this way, a sensitivity that lies in the range of ag [sic; pg] (10^{-18} g) can be achieved. Theoretically this means the detectability of individual target sequences (3-5 target sequence pieces) in the plasma pattern.

This extremely sensitive and specific detection method for the specific mRNA sequence of the substances of cancer cell origin provides a basis for a malignancy test that is suitable for diagnosis, early detection, screening and prediction of therapy outcome with antineoplastic agents, due to the special properties of malignant cells and tumors.

This test is capable not only of reflecting the fundamental molecular and genetic changes that are primarily responsible for the malignant transformation, its maintenance and progression, but also one of the most important modes of affecting the host organism by the malignant cancer cell population.

To achieve a cure, both mechanisms must be controlled:

The activated oncogenes boost the "motor" of the malignant cells, which must be "geared down" in order to be able to save the host, the patient.

The adverse effect on the host organism by the cancer cell population in the matter indicated here, which leads to weakening of the defense and other functions of the host, must be stopped.

For this, the method of the invention creates the most important prerequisites: early detection of these events in each patient individually.

Claims

1. A malignancy test (cancer test) by in vitro enzymatic amplification of the specific RNA sequence of the substances of cancer cell origin from an acellular biological liquid, in which,

a. the RNA from the acellular biological liquid is concentrated under the constant effect of a reliable RNase inhibitor,

b. the product of Step a is subjected to an in vitro reverse transcription in order to synthesize the complimentary DNA (cDNA),

c. the product of Step b is used repeatedly to synthesize and thus amplify the target-specific sequence of the desired substance of cancer cell origin enzymatically in vitro, and

d. the product of Step c is detected by determining the amplification of the nucleic acid.

2. A method as in Claim 1, characterized in that the product of Step a is subjected to in vitro hybridization with the desired oligonucleotide or the DNA probe before Step b.

3. A method as in Claim 1, which is characterized in that the in vitro amplification of the product of Step b is repeatedly carried out by a polymerase chain reaction (PCR).

4. A method as in one of Claims 1-3, characterized in that the acellular biological fluid is preferably human blood plasma.

5. A method as in Claim 1, characterized in that the specific target sequence(s) enzymatically amplify(s) two or more substances of cancer cell origin simultaneously in the same system in vitro (pooled PCR).

6. A method as in Claim 1, characterized in that, in the in vitro simultaneous amplification of a plurality of substances of cancer cell origin, each target sequence is programmed for synthesis by the appropriate choice of the primary pair and that each is synthesized in a specific characteristic size.

7. A method as in Claim 5, characterized in that during the pooled PCR, the sequences being synthesized are labeled by incorporation of primers conjugated with fluorescent dyes so that each target sequence product has a characteristic color and is correspondingly identified.

8. A method as in one of Claims 1 and 3-6, characterized in that the in vitro amplification of the nucleic acids is determined with ethidium bromide by a color reaction.

9. A method as in Claim 1, characterized in that the product of Step c is identified by its specific sequence by in vitro hybridization, by sequence determination or by scanning tunneling microscopy.

10. A method as in Claim 1, characterized in that the product of Step c is identified in correspondence with its size by gel electrophoresis or by column chromatography.

11. A method as in Claim 1, characterized in that the product of Step c in Claim 1 is already labeled during the PCR,

a. by using labeled primers, or

b. by incorporation of labeled nucleotides in which the labeling is radioisotopic or nonradioisotopic.

12. A method as in one of Claims 1, 2 and 3-9, characterized in that the in vitro hybridization is carried out,

a. with labeled DNA or oligonucleotide probes while the unlabeled target DNA or oligonucleotides amplified by PCR are either immobilized on a solid substrate or are free in solution,

b. or the target DNA or oligonucleotide amplified by PCR is labeled free in solution and the various oncogene probes are immobilized unlabeled on a solid substrate,

where a hybridization through appropriate treatment is determined by the detection of the labeling substance.

13. A method as in one of Claims 1, 2, 6, 9 and 12, characterized in that an individual DNA or oligonucleotide probe or a mixture of individual DNA or oligonucleotides is used as a probe (pooled probe).

14. A method as in one of Claims 1-6, 9, 11 and 12, characterized in that one removes the labeled probes that hybridized with the target DNA or oligonucleotide bonded to the solid carrier

in order to hybridize, rehybridize the solid substrate with the target DNA or oligonucleotide remaining on it with a new DNA or oligonucleotide probe.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DE 90/00102

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
Int. Cl.[5]	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Dialog Information Services, File 159, Cancer lit accession no. 89851433, Dobrovio A et al: "Detection of the molecular abnormality in chronic myeloid leukemia by use of the polymerase chain reaction", Blood; 72(6) 2063-5 1988	1-4
Y	WO, A1, 88/09385 (BALAZS, VIKTOR) 1 December 1988, see the whole document	1-14
Y	EP, A2, 0200362 (CETUS CORPORATION) 10 December 1986, see the whole document	1-14

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 May 1990 (07.05.90)	16 May 1990 (16.05.90)
International Searching Authority	**Signature of Authorized Officer**
European Patent Office	

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT /DE 90/00102

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A2, 0272098 (CITY OF HOPE NATIONAL MEDICAL CENTER) 22 June 1988, see the whole document —	1-14
Y	WO, A1, 89/07149 (MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH) 10 August 1989, see the whole document —	9

Form PCT/ISA/210 (extra sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DE 90/00102

SA 34427

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 88/09385	01/12/88	NONE		
EP-A2- 0200362	10/12/86	AU-B-	586233	06/07/89
		AU-B-	591104	30/11/89
		AU-D-	5532286	02/10/86
		AU-D-	5532386	02/10/86
		CA-A-	1237685	07/06/88
		EP-A-	0201184	12/11/86
		JP-A-	61274697	04/12/86
		JP-A-	62000281	06/01/87
		US-A-	4683202	28/07/87
		US-A-	4683195	28/07/87
		US-A-	4800159	24/01/89
EP-A2- 0272098	22/06/88	NONE		
WO-A1- 89/07149	10/08/89	NONE		